Chapter 4

4 Genetic evidence for the reduction of effective population size in males in a lek-breeding system

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Abstract

In lek systems, the lekking sex has a high variance in mating success which can to lead to a skew in life-time reproductive success (LRS). Sexual selection operates on the lekking sex through this variation in LRS. Many studies show a skew in annual male mating rates in lekking species, but the magnitude of the skew in life-time reproductive success (LRS) often remains unknown. However, if lekking males have a strong skew in LRS the expected ratio of maternal effective population size (N_{ef}) to biparental effective population size (N_e) is predicted to decline to $1:1/3$, instead of 1:2 for random mating (Chesser & Baker 1996). We therefore used variation in maternally transmitted mitochondrial control region sequences and eight biparentally transmitted autosomal microsatellites in the ruff *Philomachus pugnax* to estimate maternal and biparental effective population sizes with the equations $\theta = 2N_{ef}*u$ and $\theta = 4N_e*u$, respectively. Over a range of published avian and vertebrate mutation rates (μ) the ratio between the maternal and bi-parental effective population size was <1:1. This confirms that in ruffs the mating bias among males ultimately results in a skew in LRS larger than that among females. This skew in LRS explains the strong sexual selection on male ruffs.

Introduction

Lek mating systems produce large variance in individual mating success of the lekking sex (Mackenzie *et al.* 1995). An unsolved issue in studies of leks is the extent to which the annual or apparent skew in mating success (Kokko *et al.* 1998, 1999) ultimately produces comparable skews in life-time reproductive success (LRS) (Widemo 1998). Variance in LRS enables sexual selection to operate and will result in sexual dimorphism (Andersson 1994). In the buff-breasted sandpiper *Tryngites subruficollis*, a lekking species with limited sexual dimorphism, a male mating skew at leks was not mirrored by a skew in paternity; many males achieved paternity by copulating outside leks, considered 'exploded' leks in this species (Lanctot *et al.* 1997). To determine whether species with more developed lek systems than buff-breasted sandpipers and hence stronger sexual selection, have unequally skewed LRS between the sexes, behavioural observations and paternity analyses are needed. However, these measures are difficult to obtain in non-monogamous species and are usually limited to a selection of individuals followed for a limited proportion of their reproductive life (but see Duval & Kempenaers (2008)).

Evolutionary histories of variance in LRS can also be detected by the current genetic variation in a species. Unequal mating success leads to a smaller effective population size (N_e) than random mating (Chesser & Baker 1996; Emlen & Oring 1977). Under random mating, the theoretical difference in the maternal and biparental effective size is close to N_{ef}:N_e = 1:2 (Chesser & Baker 1996). However, under the scenario of a strong male skew in LRS, the lek model of Chesser and Baker (1996) predicts that the ratio N_{ef}:N_e can be as low as 1:$^1/_3$. A scenario of a sex-specific skew in mating would thus lead to differential effects on N_e in sex-linked versus biparentally inherited genetic markers. A comparison of N_e estimated with sex-linked genetic markers that are only transferred by half the individuals, in comparison with N_e estimated with bi-parental markers, may help us establish any sex-related skews in LRS (Chesser & Baker 1996).

Here we assess the skew in LRS of male ruffs (*Philomachus pugnax*) by estimating how the ratio N_{ef}:N_e deviates from the random mating model. Ruffs are long-distance migratory shorebirds with a strong and complex lek mating system (Hogan-Warburg 1966; Jukema & Piersma 2006; van Rhijn 1973, 1991; Widemo 1998). Variance in female LRS is expected to less strongly skewed than in males as it largely is a function of survivorship, since clutch size is almost unimodal and double brooding is unlikely (Piersma *et al.* 1996). In contrast, many male ruffs don't mate at all in some years (e.g. Hogan-Warburg 1966) and thus variance in male LRS is expected to be high, especially since three genetic types of males persist: independents, satellites and faeders (Jukema & Piersma 2006; Lank *et al.* 1995). Despite extensive evidence of annual skew in mating success between and within these permanent mating strategies (see below), it has not been tested whether this leads to a skew in LRS that is strong enough to affect genetic variation.

Independents make up 80-90% of male ruffs on leks. They are large, colourful and feisty, and can behave as residents, who display and mate on territories on the lek, or

as marginals, who rarely mate at leks (Hogan-Warburg 1966; van Rhijn 1973, 1991). Satellites are recognised by white or light plumage and share territories with residents (Hogan-Warburg 1966). They occur in lower numbers than independents (10-20%), consistent with their lower mating success as documented in the field (Widemo 1998). Faeders are females mimics; they develop no sexual ornaments, are only slightly larger than the small females (Jukema & Piersma 2006). Their mating system is still unknown, but they are able to sire offspring in captivity (D.M. Lank & S. McRae, unpubl. data). Faeders occur at a frequency of about 1% of populations (Chapter 5).

The differential mating rates within and among independents, satellites, and faeders is expected to ultimately lead to a skew in male LRS. However, variation in LRS could be smaller than commonly assumed (Widemo 1998). Firstly, males unsuccessful in a concurrent year might be investing in future reproduction. Secondly, over half of the clutches in ruffs have multiple fathers (Lank *et al.* 2002; Thuman & Griffith 2005). Multiple paternity might reduce the skew in polygynous species (Webster *et al.* 1995) unlike in monogamous species (Dolan *et al.* 2007). Thirdly, the skew in mating success decreases with lek size (Höglund *et al.* 1993; Höglund *et al.* 1998; Widemo & Owens 1995), but see Kokko *et al.* (1998), so when most males operate on larger leks (>20 males) the mating skew might be limited (Widemo 1998). Fourth, mating skews observed at leks might be balanced by off-lek copulations (Lank & Smith 1987; van Rhijn 1991). In paternity studies a proportion of the offspring could not be assigned to fathers (Lank *et al.* 2002), hinting at copulations at other leks with unsampled males or off-lek copulations.

Behavioural studies examining a possible skew in LRS of ruffs have not been able to estimate the magnitude of true skew in LRS. A useful alternative approach in assessing skew in LRS is through its effect on genetic variation in maternally and biparentally transmitted genes. Confounding factors when assessing sex-specific skew in LRS by determining genetic variation of sex-linked markers are (1) sex-specific dispersal rates, (2) unequal sex ratios and (3) sex-specific population fluctuations. Hence, in addition to N_e and N_{ef}, sex-biased dispersal was estimated and effects of sex ratio distortion were modeled.

Methods

Sample collection

Blood and tissue samples of ruffs were collected in breeding areas in Sweden (by K.A. Thuman and F. Widemo), Finland (by D.B. Lank), Gydan, Taymir, Yakutia and Chukotka (by M.Y. Soloviev and P.S. Tomkovich *et al.*). Blood samples were stored in 98% ethanol at –80°C. All samples, except the Swedish samples, are curated in the collections of the Royal Ontario Museum, Toronto, ON, Canada and the University of Groningen Shorebird LifeLines collection. DNA was isolated with a DNeasy Blood and Tissue Kit (Qiagen) or by standard phenol–chloroform extractions, and stored at –20°C.

Mitochondrial (mtDNA) control region

A segment of Domain I & II of the control region was amplified and sequenced with forward primer L141 (5′- TCCATTAATCTACAACCGGGCT) and reverse primer PropR (5′-AATACCAGCTTTGGGAGTTGG). By anchoring the primer PropR in $tRNA^{Pro}$ at the end of the first control region the first CR only was targeted (ruffs have two full duplicated copies of the control region, Chapter 3). The amplification profile used was 2 min denaturation at 95°C, followed by 36 cycles of 94°C for 45 s, 53-57°C for 45 s, 72°C for 1.30 min, followed by a final 7 min elongation at 72°C. PCR products were gel-purified, prepared for sequencing using BigDye Terminal Cycle Sequencing reagents according to the manufacturer's instructions (Applied Biosystems, Foster City, USA) and sequenced on an ABI 3100 automated sequencer. Sequences were edited and aligned in MEGA 3.1 (Kumar, Tamura & Nei 2004). Control region I sequences of 512 nt from domain I and II were obtained for 63 individuals from various breeding locations. The transition/transversion bias in this sample of breeding birds was R = 33. See Chapter 3 for further information on sequence variation.

Nuclear (nDNA) microsatellites markers

A total of 66 individuals from various breeding locations were genotyped for eight autosomal polymorphic microsatellites (*ruff1, ruff6, ruff10, ruff12, ruff50, KN2, Phil9, SN13*). The markers were published loci cloned from ruffs (Thuman *et al.* 2002) and three novel loci which were isolated from a red knot, a great snipe library and a ruff genomic library provided by O. Haddrath (pers. comm.) (KN2: forward primer sequence (F) (5′-3′): ACATGCAAATTCACGCCCCAG; reverse primer sequence (R): TACCCTG-CAAATGACAGAAAGGGCT SN13: F:TGTCATGTTTAGCTTGGGCT; R:AGGCTG-CAACTCCGCAGCAC; and Phil 9: F:GACCACCCAAAGCCCTATAA; R:TTTCTTTTT GAATTCACTAGCCATC). Sequences used to design primers for these three loci have been deposited in GenBank.

PCR was carried out on a Mastercycler epgradient S (Eppendorf). PCR reaction volume was 12.5 µL, containing 1x PCR buffer (10 mM Tris-HCL pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.01% gelatin), 0.7 pmol dNTP (Invitrogen), 0.75 pmol forward primer (Invitrogen), 2.5 pmol reverse primer (Invitrogen), and 2.5 pmol universal dye-labelled M13 (TGTAAAACGACGGCCAGT) tail (6-FAM, NED, VIC; Applied Biosystems), 0.25 U Taq (Invitrogen) and 1.0 µL DNA template. PCR conditions included an initial denaturation step at 94°C for 5 min, 36 cycles including denaturation at 94°C for 30 s, primer annealing at 54 or 57°C for 30 s, and primer extension at 72°C for 30 s. A final step at 72°C for 5 min was used to complete primer extension. Fragment analysis was run on the ABI 3100. Alleles were sized using 500LIZ size standard (GeneScanTM), and allele sizes were assigned with Genemapper 3.7 (Applied Biosystems).

Estimating effective population size using Coalescent analysis

We inferred the effective population sizes for mtDNA and nDNA markers using Coalescent analysis (Beerli & Felsenstein 1999, 2001; Rosenberg & Nordborg 2002). The coalescent is able to estimate population parameters for non-equilibrium populations. To

calculate effective population sizes the variance parameter θ was estimated for both markers. The effective population size was calculated using $\theta = 2N_{ef}*\mu$ for mtDNA and $\theta = 4N_e*\mu$ for nuclear DNA, where μ is the mutation rate. To estimate θ the program LAMARC (Kuhner 2006) was used which allows a single, non-equilibrium population (Excoffier & Heckel 2006). The haplotypes/genotypes were considered to be sampled from a genetically uniform global population (Chapter 3). For all runs, curve-files were plotted to assess the shape of the posterior probability distributions.

For microsatellites, the Bayesian brownian motion model was used, which is a continuous modification of the discrete stepwise mutation model. Six short initial runs were performed to assure that estimates converged and to determine how many trees needed to be sampled to obtain unimodal posterior probabilities. One long run was executed with priors for θ set at 0.001-30, which sampled 50,000 trees, with sampling increments of 100. Adaptive heating was set to three, meaning that the parameter space was sampled by three simultaneous chains. For μ, we assumed a range of possible mutation rates of $0.001\text{-}1\times10^{-5}$ mutations per locus per generation, around the typical vertebrate mutation rate of 1×10^{-4} s/l/generation, taken from fig. 3 in Whittaker *et al.* (2003) where mutation rates of microsatellites for repeat lengths are given for mammals. Birds have fewer microsatellites than mammals but characteristics such as length, allele dispersion and range of allele sizes do not vary between birds and mammals, indicating that mutation rates are comparable (Neff & Gross 2001). For calculation of N_e a generation time of 3 years was assumed.

For mtDNA sequences, the F84 Bayesian model to correct for multiple hits was used (Kuhner 2006), with the transition/transversion bias set to 33. To test whether the estimates converged, seven short initial runs were performed, sampling 20–50,000 trees and using default priors ranging between 0.00001-10. In some runs, the autocorrelation was set to 60 to control for the tendency of mutation rates to "clump", but this did not affect estimates. The final run had three simultaneous chains, using priors for θ (q1) set to 100 and for M to 100. Runs were extended until effective sample size for all parameters was > 50. For μ we assumed a range of possible mutation rates around 2–15% per Myr, which translates into $2\text{x}10^{-8}$ to 1.5×10^{-7} per locus per year (our locus being 512 bp). These estimates include values for shorebirds for Domain I of the control region (Wenink & Baker 1996) and also the lower mutation rate for sequences including Domain II (Buehler & Baker 2005).

Testing for sex-biased dispersal

The Chesser and Baker (1996) model assumes high female and low male dispersal, which is a common feature in lekking species (Bouzat & Johnson 2004; Francisco *et al.* 2007; Gibson *et al.* 2005; Höglund *et al.* 1999; Lebigre *et al.* 2008; McDonald 2009; Regnaut *et al.* 2006; Segelbacher *et al.* 2007). In male ruffs, high adult lek site fidelity has been observed (Emlen & Oring 1977; Widemo 1997). However, migratory males have shown large flexibility in migration routes, including breeding destinations, suggesting that dispersal may occur (Chapter 3). Also females might have lower dispersal than the model assumes (but see Andersen (1948, 1951)).

To examine dispersal characteristics, sex-biased dispersal was estimated by comparing population parameters estimated separately with biparental and maternal markers.To test for sex-biased dispersal, 66 microsatellite genotypes between males (n = 39) and females (n = 27) were compared, using the biased dispersal option in FSTAT (Goudet 2001; Goudet *et al.* 2002). Weir and Cockerham's θ (Weir & Cockerham 1984) was used as a differentiation parameter as it is more powerful and less sensitive to the magnitude of sex-biased dispersal (in the Results this θ will be denoted as F_{st}, to avoid confusion with the demographic parameter theta (θ)). The FSTAT biased dispersal tests returns a measure of relatedness (F_{st}) between samples and a measure of sub-structuring within samples, which is the Wahlund effect (F_{is}). Under the hypothesis that males have high philopatry they should display higher F_{st} and a lower F_{is} than females (Goudet *et al.* 2002; Prugnolle & de Meeus 2002).

Model for expected ratio N_{ef}:N_e

As we estimated N_{ef} and N_e with the maternally inherited mtDNA markers and with biparentally inherited nDNA markers, this would change the ratio N_{ef}:N_e = 1:2 predicted by Chesser and Baker (1996) to N_{ef}:N_e = 1:4 as mitochondrial markers are haploid. However, N_e and N_{ef} were calculated using the coalescent in LAMARC as $\theta = 2N_{ef}*\mu$ for mtDNA and $\theta = 4N_e*\mu$ for nDNA, and these equations account for ploidy differences. Note that the factor two in both equations accounts for the fact that in coalescent analyses θ is estimated over two branches of the ancestral tree (Kuhner 2006) (see http://evolution.genetics.washington.edu/lamarc/documentation/forces.html). More importantly, the Chesser and Baker model was not constructed for a case with unequal sex ratios, hence the model needed to be adapted. As adult sex ratio in ruffs is approximately 40% males to 60% females (Jaatinen *et al.* 2010; Zwarts *et al.* 2009), the expected N_{ef}:N_e under a random mating scenario is $1:(2\cdot 2/3)$, which is $1:1\,1/3$. Any values lower than $1:1\,1/3$ would indicate that male ruffs have a skew in LRS (Fig. 4.1).

Results

Effective population size

For the maternally inherited control region, θ_{ef} was 0.029 (95% CI 0.019-0.042). For biparentally inherited microsatellites, θ_e was 8.7 (95% CI 3.9-9.6). θ_{ef} had a unimodal distribution of posterior probabilities and was very similar to estimates from seven initial runs (0.029±0.007). Regarding θ_e, initial shorter runs revealed slightly higher estimates of 8.97 (±1.86), but multi-modal posterior distributions indicated that run lengths were insufficient. The final run yielded unimodal posterior probabilities distributions for the overall θ_e and for the θ_e for each microsatellite marker.

To convert θ to effective population size, a range of mutation rates for avian control region sequence and vertebrate microsatellites was accounted for. Over this range of mutation rates, the ratio N_{ef}:N_e was just below 1:1 (Fig. 4.2). Note that this comparison

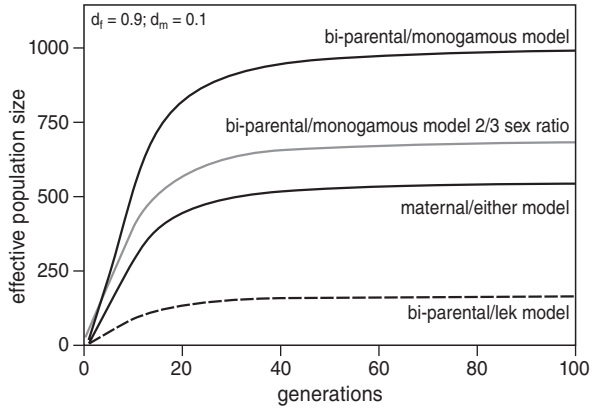

Figure 4.1. The Chesser and Baker (1996) predictions for effective population size under two avian mating systems. The original model considers equal sex ratios, and is here modified for a biased sex ratio. (Solid lines) Monogamous mating system, where male polygyny (Φ_m) is zero; (Dashed line) Lek mating system, with high male polygyny (Φ_m = 0.9). In both models high female dispersal (d_f) and low male dispersal (d_m) is assumed. For explanation of model and model parameters see Chesser and Baker (1996).

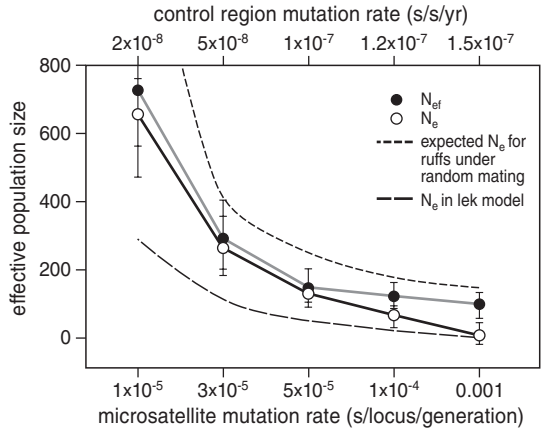

Figure 4.2. Effective population size estimates as derived from biparental nuclear (N_e) and maternal genetic markers (N_{ef}) under the range of published vertebrate mutation rates. The expected effective population size under random mating and under a lek model, are presented for comparison. Vertical lines indicate 95% CI of estimates. The most likely estimate for N_{ef} is at a mutation rate of 5×10^{-8} s/s/yr (5% s/Myr), determined for the control region in shorebirds (Buehler & Baker 2005). The most likely estimate for N_e is at a mutation rate of 1×10^{-4} s/l/generation, a commonly used vertebrate mutation rate (Neff & Gross 2001; Whittaker *et al.* 2003). (Note that a generation time of 3 years was used to calculate N_e.)

is conservative with regard to detecting lower N_e than N_{ef}, as relatively high mutation rates were assumed for the control region and relatively low mutation rates for microsatellites.

Sex-bias in genetic structure

The sex-biased dispersal statistics revealed that relatedness within samples for both males and females was not significantly different from zero ($F_{st\text{-}males}$ = 0.010 (95% CI -0.009-0.033; n = 39) and $F_{st\text{-}females}$ = 0.009 (95% CI -0.016-0.045; n = 27). In females, F_{is} was significantly different from zero ($F_{is\text{-}females}$ = 0.17 (95% CI 0.07-0.32)), but not in males ($F_{is\text{-}males}$ = 0.059 (95% CI -0.03-0.12)).

Discussion

In ruffs, biparental effective population size (N_e) was reduced relative to female effective population size, indicating that male effective population size (N_{em}) is reduced. This is not the first study assessing N_e in a lekking species, but it is the first to detect a sex difference in N_e. In the Gunnison sage-grouse (*Centrocercus minimus*), a ground-lekking bird, N_{ef} and N_{em} were similar, as the variance in seasonal reproductive success was almost as high in females as in males, due to a high rate of nest failure (Stiver *et al.* 2008). Similarly in the lek-breeding European treefrog (*Hyla arborea*), no sex difference in effective population size was found; a weak negative effect of the mating system was obscured by the much stronger effect of delayed maturity in both sexes (Broquet *et al.* 2009). Sexual selection theory states that in lek breeding species the reduction in N_e of the lekking sex is a consequence of larger reproductive variance and sexual selection in the lekking sex (Chesser & Baker 1996). Is our finding that ruffs have a reduced N_e consistent with the lek model and does this imply that in ruffs variance in male LRS drives sexual selection?

The observed ratios for N_{ef}:N_e was just below 1:1. Since even the 95% CI for N_e did not reach the theoretical ratio N_{ef}:N_e = 1:1$^1/_3$ expected under a scenario of random mating and 2:3 sex ratio, we reject random mating in ruffs. Note that a scenario of equal sex ratios the expected Nef:Ne is 1:2. The observed ratio Nef:Ne was below 1:1, which is not as low as the lek model predicting N_{ef}:N_e = 1:$^1/_3$. However, the lek model assumes high female dispersal (d_m = 0.9, Fig. 4.1). Genetic structure analysis could not confirm a strong female-bias in dispersal, so this assumption might not apply to ruffs. If on the contrary ruffs had high male dispersal and low female dispersal, the ratio N_{ef}:N_e would be larger than 1:1 (fig. 2a in Chesser and Baker (1996)). This would indicate that in ruffs the relative differences between dispersal of males and females might be smaller than assumed in the model, but that stronger female dispersal still applies. Indeed, a significant Wahlund effect in females was found which indicates higher dispersal tendencies in females than in males (Goudet *et al.* 2002; Prugnolle & de Meeus 2002). Hence we conclude that the lek model of Chesser and Baker (1996) assuming more female dispersal and more male philopatry could apply to ruffs. Note that this does not imply that philopatry in male ruffs is as strong as in other lekking species; for leks to evolve, male philopatry is not a prerequisite because female choice for male aggregation alone is enough (Kokko 1997; Pruett-Jones & Pruett-Jones 1990).

A reduction in male effective population size is partly a consequence of a male skew in reproductive success, but is also the product of demographical differences between the sexes. We adjusted the lek model of Chesser and Baker (1996) for a skew in overall adult sex ratio. Ruffs on a population level have approximately 60% females (Zwarts *et al.* 2009). Throughout Africa, where the majority of the global population winters, males contribute 7-52% to the population, e.g. in the Sahel, where 1–2 million of this population winters (Delany *et al.* 2009), the proportion of males is 36% (OAG Münster 1996; Zwarts *et al.* 2009). Additionally, the female:male ratio in juveniles arriving from the breeding areas in staging sites is close to 60:40 (Jaatinen *et al.* 2010). Although the female-biased sex ratio was accounted for in the model predicting the reduction in biparental effective population size, a bias of 60:40 could not fully account for the magnitude of the reduction in N_e. The female-biased sex ratio is itself a likely concomitant of strong sexual selection in the lek breeding system (Wade *et al.* 2003; Wade & Shuster 2003).

Evidence for strong reproductive skew in ruffs confirms the intuition of previous researchers who observed the gigantic investment of some males in dominating the lek (Hogan-Warburg 1966; van Rhijn 1983, 1991): independent males may display so intensively that they refrain from eating and drinking, and lose mass throughout the breeding season (Bachman & Widemo 1999; Widemo 1998) (pers. obs.). Our study suggests that such investments do pay off in relatively high LRS. Since females have extensive choice, their preferences largely determine male effective population size in ruffs. If female choice is directed towards 'good genes' then directional selection could reduce genetic variation even further (Andersson 1994). This means that forces of sexual selection act strongly on male ruffs.

To generalize our findings, future studies should test whether the observed reduction in male effective population size in ruffs is a unique feature of lekking species and/or whether this reduction also occurs in other mating systems. Shorebirds are a suitable group to test the hypothesis that the strength in sexual selection is related to a reduction in male effective population size, because a large variation in mating systems exists in this clade (Piersma *et al.* 1996; Thomas *et al.* 2007). In this study, multiple autosomal loci and a single mtDNA locus were suitable markers because in ruffs variation in mtDNA is not constrained (Chapter 3), however in other species genetic variation in mtDNA might be reduced due to genetic sweeps. Future studies should use multiple unlinked loci on the Z-chromosome instead of a single mtDNA marker, as suggested before (Ellegren 2009; Mank *et al.* 2010; Mank *et al.* 2010).

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